To the Graduate Council:

I am submitting herewith a thesis written by Christopher Aaron Kiefer entitled "Detection and Enumeration of *Escherichia coli* O157:H7 and *Yersinia enterocolitica* from Farm Animal Environments." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Science and Technology.

David A. Golden, Major Professor

We have read this thesis and recommend its acceptance:

Accepted for the Council:

Associate Vice Chancellor and
Dean of The Graduate School

DETECTION AND ENUMERATION OF *Escherichia coli* O157:H7 AND *Yersinia enterocolitica* FROM FARM ANIMAL ENVIRONMENTS

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Christopher Aaron Kiefer
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DEDICATION

This thesis is dedicated to my parents, Dave and Melissa Kiefer and my sister Keri, whose love and support are deeply treasured.

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ABSTRACT

Recovery of *Escherichia coli* O157:H7 and *Yersinia enterocolitica* from farm samples (feed, bedding, fecal samples, and water) from various animal sources (cattle, swine, and chickens) was investigated at inoculum levels of 10^4 CFU/g (high level) and 10 CFU/g (low level) of sample. High-inoculum levels of *E. coli* O157:H7 were recovered using direct plating on sorbitol MacConkey agar (SMAC), SMAC supplemented with cefixime and tellurite (CT-SMAC), hemorrhagic colitis agar (HC), modified eosin methylene blue agar (MEMB), and Rainbow® agar O157. Low-inoculum levels of *E. coli* O157:H7 were recovered using a 24-hr enrichment at 37°C in modified tryptic soy broth supplemented with 0.02 g/l novobiocin (mTSB+N), EHEC enrichment broth (EEB), and modified *E. coli* broth supplemented with 0.02 g/l novobiocin (mEC+N) followed by streaking on to SMAC, CT-SMAC, HC, MEMB, and Rainbow® agar O157. In addition, low-inoculum levels of *E. coli* O157:H7 were detected using immunomagnetic separation (IMS), EHEC-Tek®, and Reveal® for *E. coli* O157:H7 test systems. High-inoculum levels of *Y. enterocolitica* were recovered using MacConkey agar (MAC), cefsulodin-irgasan-novobiocin agar (CIN), and KV-202 agar. Low-inoculum levels of *Y. enterocolitica* were recovered using cold enrichment in peptone sorbitol bile broth (PSBB) followed by streaking on to MAC, CIN, and KV-202.

Eight sample types were evaluated. For direct plating, three repetitions were performed, giving a total of 24 (8x3) inoculated samples tested. Duplicate sample types were examined for low inoculum evaluations, also performed in triplicate, for a total of 48 (8x2x3) individual samples evaluated. Rainbow® agar O157 recovered high-inoculum levels of *E. coli* O157:H7 by direct plating from the most sample types (7 of 8), but

provided the poorest numerical recovery in 6 of 7 sample types from which the organism was recovered ($P<0.05$). CT-SMAC and Rainbow® agar O157 recovered *E. coli* O157:H7 from 18 of 24 individual samples. CT-SMAC and MEMB recovered *E. coli* O157:H7 by direct plating from 6 of 8 sample types. MEMB provided the best numerical recovery in total mixed ration cattle feed (TMR), cow fecal, and pig feed sample types ($P<0.05$). Using enrichment, mTSB+N followed by streaking on to CT-SMAC provided the best recovery (18 of 48 samples tested) of low inocula. The EHEC-Tek® test system provided positive results in 18 of 24 inoculated samples, with chicken fecal/bedding mixture not providing a positive result (0 of 3). The Reveal® for *E. coli* O157:H7 test system provided positive results in 20 of 24 inoculated samples, however false positives in uninoculated samples were encountered with regularity. IMS performed best when preparations were plated on to Rainbow® agar O157 (10 of 24 positive), but the method was inferior to EHEC-Tek® and Reveal® for *E. coli* O157:H7 for recovery of low-inoculum levels ($P<0.05$).

High-inoculum levels of *Y. enterocolitica* were recovered from the most sample types with CIN (7 of 8), followed by KV-202 (6 of 8), and MAC (5 of 8). CIN agar provided superior numerical recovery over KV-202 in TMR, pig fecal, chicken fecal, and pig feed sample types ($P<0.05$), but test media did not differ significantly in their ability to recover the organism from the pig feed sample type (46 indivdual samples evaluated) ($P<0.05$). After enrichment, streaking to CIN provided the best recovery (19 of 46), followed by MAC (13 of 46), and KV-202 (11 of 46).

These results indicate that some direct plating methods used for isolation of *E. coli* O157:H7 and *Y. enterocolitica* from foods may be satisfactory for recovery of high

levels of these pathogens from farm animal environments. While enrichment alone did not allow for consistent recovery of low levels of *E. coli* O157:H7 from such environments, EHEC-Tek® and Reveal® for *E. coli* O157:H7 test systems provide good recovery (18 and 20/24 samples respectively). Cold enrichment in PSBB is an unreliable method for recovery of low inocula of *Y. enterocolitica* from farm animal environments.

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Part I:

Literature Review

INTRODUCTION

There is a link between farm environments and transmission of pathogenic microorganisms to food. An outbreak of *Escherichia coli* O157:H7 associated illness associated with lettuce consumption in Montana in 1995 was traced to composted dairy manure (Ackers et al., 1998). This case illustrates comparative ease at which ready-to-eat foods such as fresh produce can become a vector of foodborne illness. Feces, soil, irrigation water, inadequately composted manure, dust particles, wild or domestic animals, and humans can all be sources of contamination (Beuchat, 1996). A wide range of microorganisms including *Shigella* spp., *Salmonella* spp., *Escherichia coli*, *Campylobacter* spp., *Yersinia enterocolitica*, *Aeromonas* spp., *Listeria monocytogenes*, *Staphylococcus aureus*, spore-forming bacteria, as well as viruses can be associated with contaminated fresh produce (Beuchat, 1996).

In addition to fresh produce, the farm to food infection mechanism can be seen in the contamination of meat by contact with intestinal contents during the slaughter process. Miller et al. (1997) described a combination of distended intestines (feed presence) and stress of handling as a cause of either small ruptures in the intestines or increase the likelihood of accidental puncture of the intestinal lining during evisceration of swine, leading to an increased risk in contamination.

E. COLI O157:H7 AND *Y. ENTEROCOLITICA* IN THE FARM ENVIRONMENT

Feeds

It has been theorized that animal feed can be an important link in the transmission of pathogenic bacteria from one animal to another. There is little done in the United

States to monitor bacterial quality of animal feeds (Hancock et al., 1997). A study by Lynn et al. (1998) found that 30% of cattle feeds were positive for *E. coli*, an indication of fecal contamination. The study found no feed sample positive for *E. coli* O157, however *E. coli* O157 inoculated into cattle feeds at an initial population of 3 $\log_{10}$ CFU/g reported a -2.0 to 5.0 $\log_{10}$ CFU/g change in populations. Fecal shedding of *E. coli* O157:H7 in herds of cattle has been shown to have periods of high shedding followed by little or no shedding, suggesting a common source of exposure, such as a feed trough. (Hancock, 1997b).

Manure

Fecal material has been shown to be an important vehicle for transmission of *E. coli* O157:H7 and *Y. enterocolitica*. Chapman et al. (1997) found that *E. coli* O157 was isolated from the feces of 15.7% of cattle. The research indicated that, among various farm animals tested, *E. coli* O157:H7 was most commonly associated with cattle. An important aspect of *E. coli* O157:H7 presence in cattle is that the organism is not pathogenic to cattle, so visual inspection of the animal is not a option to detect the organism (Whipp et al., 1994). A study investigating the presence of *E. coli* O157 at 36 different cattle farms detected the organism in 27 of the tested sites (75%). The same study found that 1.4% of fecal samples taken were culture positive for *E. coli* O157 (Hancock, et al. 1997b). Rice et al. (1997) found that 7.9% of fecal samples of cull cows tracked from farm to slaughter were positive for *E. coli* O157. Hancock et al. (1997a) found that 1.8% of cattle fecal samples and 63% of cattle feedlots were positive for *E. coli* O157 and concluded that: (1) the organism was widely distributed throughout the United States, and (2) the prevalence in herds was low. Bonardi et al. (1999) found that

13% of fecal samples from cattle at slaughter tested positive for verocytotoxin-producing *E. coli* O157 (VTEC). The study revealed that feedlot cattle and dairy cull cattle were positive for the organism at a higher overall rate (approximately 16%) than veal calves (0%). It has been revealed through epidemiological data that populations of *E. coli* O157:H7 in cattle reservoirs range from $<10^2$ to 10^6 CFU/g (Zhao et al., 1995). *E. coli* O157:H7 inoculated into cattle manure at a population of 10^5 CFU/g survived for 70, 56 , and 49 d when incubated at 5, 22, and 37°C, respectively (Wang et al., 1996). Kudva et al. (1998) demonstrated that, when waste effluents from bovine farms were inoculated with *E. coli* O157:H7 and incubated at low temperatures (≤23°C), the organism remained the predominant bacterium recovered. However, when the effluent was incubated at 37 or 45°C, the normal background flora was predominant in the culture. The same study revealed that *E. coli* O157:H7 survived in sheep manure for 12 months.

Chapman et al. (1997) found that 0.4% of rectal fecal samples of 1000 pigs were positive for *E. coli* O157, although these isolates were not verocytotoxigenic. Three isolates were non-motile, and one was motile but did not express the H7 antigen. Chapman concluded that these strains were probably atypical and unlikely as a source of infection.

Chickens can be colonized by *E. coli* O157:H7 without disease symptoms, but evidence of *E. coli* O157:H7 presence on chicken farms is scarce. Of 50 poultry farms surveyed, the *E. coli* O157:H7 was not detected among any of 500 birds tested (Doyle et al, 1997). The organism has been shown to infect chickens and remain in their gastrointestinal systems for a substantial period of time. Beery et al. (1985), found that *E. coli* O157:H7 can remain in the ceca from 4 to 28 d and can be excreted in the feces

for up to 90 d. Stanley et al. (1996) reported that when chicks that were challenged with 10^9 *E. coli* O157:H7, the organism was unrecoverable after 21 d. It has been shown that 1 to 3% of wild bird feces is contaminated by *E. coli* O157 (Wallace et al., 1997). However, a study by Jeffery et al. (1998) found no *E. coli* O157 on the surface or inside of dried poultry litter piles, which were intended for use in cattle feed.

Y. enterocolitica serotypes that are most commonly associated with human disease are most frequently isolated from swine (Kapperaud, 1991). Thibodeau et al. (1999) reported that *Y. enterocolitica* was isolated from 5.8% of feces from pigs at slaughter. Shiemann (1982) found the organism in 19 to 29% of fecal samples of pigs slaughtered and processed using three different evisceration methods. The results of the study indicated that contamination of the carcass was related to the evisceration technique that allowed the least amount of fecal contamination. Fantasia et al. (1993) found that 40% of fecal samples isolated were positive for *Y. enterocolitica*, although none of the isolates were of a pathogenic serotype. Letellier et al. (1999) reported that *Y. enterocolitica* was found in 21% of chicken cecal samples tested, and the most frequent serotypes found were O:3 (86%) and O:5 (9%). This study also indicated a seasonal difference in *Y. enterocolitica* presence, with the organism present more often in intestinal contents in the winter than in the summer.

Y. enterocolitica does not produce disease symptoms in cattle when the animals are fed large doses of the organism. Garin-Bastuji et al. (1999) reported that 46 consecutive doses of over 10^9 CFU of the organism produced no noticeable signs of infection, thus increasing the importance of monitoring fecal levels of *Y. enterocolitica*. Davey et al. (1983) investigated the presence of *Y. enterocolitica* in fecal samples of

cattle. *Y. enterocolitica* or related species were isolated from 50% of the animals tested, with several strains being associated with human illness. Fantasia et al. (1993) isolated non-pathogenic *Y. enterocolitica* from 42% of bovine fecal samples. Hughes (1978) detected *Y. enterocolitica* in cattle manure at populations of 5×10^3 CFU/g of fecal material.

Water

Water has been implicated as a vector for *E. coli* O157:H7 in several instances (MMWR, 1996a; MMWR, 1996b). A 1992 outbreak involving water resulted in four deaths among 243 documented cases. The source of the contamination was never specifically identified, but it was theorized that replacements of water meters or line breaks were the cause (Geldreich et al., 1992). Warburton et al. (1998) demonstrated that *E. coli* O157:H7 survived for >300 days in sterilized bottled water. Rajkowski and Rice (1999) reported that *E. coli* O157:H7 survived and grew in unchlorinated, reconditioned pork-processing wastewater.

Y. enterocolitica has been shown to survive for up to five days in river water inoculated with 10^7 CFU/ml and for three days in river water inoculated with 10^5 CFU/ml of the organism (Chao et al., 1988). The same research showed no decrease in *Y. enterocolitica* populations in physiological saline (0.85% NaCl) and found that the organism had variable survival in ground water, ranging from a 10^4 to no decrease after 10 days. Terzieva and McFeters (1991) showed that *Y. enterocolitica* survived best at 6 and 16°C in agricultural surface water and concluded that the water might serve as a vehicle for transmission of the bacteria between animals and humans. Karapinar and Gonul (1991) reported that *Y. enterocolitica* could survive up to 64 weeks in sterile

spring water with no reduction in population.

RECOVERY METHODS FOR *E. COLI* O157:H7 AND *Y. ENTEROCOLITICA*

Plating Media

There are various selective media available for detection of *E. coli* O157:H7 and many involve sorbitol utilization as a diagnostic tool. Lack of sorbitol fermentation within 24 hr has been noted as a phenotypical characteristic of *E. coli* O157:H7, and sorbitol MacConkey agar (SMAC) has been used to exploit this characteristic to detect the organism (March and Ratnam, 1986). Krishnan et al. (1987) found that SMAC was an effective plating medium for isolation of *E. coli* O157:H7 from human fecal material. Padhye and Doyle (1991) noted similarity of *Enterobacter*, *Proteus*, and *Hafnia* colonies to *E. coli* O157:H7 on SMAC made isolation of *E. coli* O157:H7 from raw milk difficult. Rajkowski and Rice (1999) reported that tryptic soy agar (TSA) recovered *E. coli* O157:H7 better than SMAC in reconditioned pork processing water. The addition of cefixime and tellurite to SMAC (CT-SMAC) has been used to increase selectivity of the medium for *E. coli* O157:H7 (Zadik et al., 1993). This medium has been shown to inhibit non-sorbitol fermenting bacteria such as *Aeromonas*, *Plesiomonas*, *Morganella morganii*, *Providencia*, and *Hafnia alvei* (Weagent et al., 1995). A study by Onoue et al. (1997) found that CT-SMAC provided better recovery of *E. coli* O157:H7 from minced beef than SMAC, sorbitol IPA bile salt agar, or Rainbow® agar O157.

Another medium that exploits the inability of *E. coli* O157:H7 to ferment sorbitol is hemorrhagic colitis agar (HC). HC incorporates 4 methyl-umbelliferyl-beta-D-gluconuride (MUG) reagent, allowing for differentiation of organisms that possess the

enzyme β-glucuronidase and those that do not by observing fluorescence (MUG-positive) under ultraviolet light. Hammack et al. (1997) concluded that HC and SMAC did not differ in ability to recover stressed or unstressed *E. coli* O157:H7 from whole milk and ice cream. However, HC recovered unstressed *E. coli* O157:H7 from Brie cheese better than SMAC. In two studies using various food samples, Feldsine (1997a; 1997b) noted that nondistinctive characteristics of *E. coli* O157:H7 on HC after enrichment in EHEC enrichment broth necessitated that a large number of colonies be picked for confirmation.

Modified eosin methylene blue agar (MEMB) utilizes sorbitol fermentation as the means of colony differentiation, and incorporates novobiocin as a selective agent. Clavero and Beuchat (1995) found that recovery of *E. coli* O157:H7 from ground beef and salami was better on MEMB than SMAC supplemented with MUG. Taormina et al. (1998) reported that MEMB and Rainbow® agar O157 recovered significantly higher numbers of heat-stressed *E. coli* O157:H7 from ground beef than CHROMagar® O157, SMAC, CT-SMAC, and SMAC supplemented with cefixime and rhamnose (CR-SMAC). These researchers attributed the superior recovery to the level of selectivity that allowed for inhibition of background microflora, while not interfering with recovery of the stressed cells.

Rainbow® agar O157 utilizes chromogenic substrates specific for two enzymes that *E. coli* possesses. Activity of β-galactosidase and β-glucuronidase produces blue-black and red colonies on the medium, respectively (Stein and Bochner, 1998). EHEC strains produce distinctive black or gray colonies on Rainbow® agar O157 due to the absence of β-glucuronidase. Colonies of other strains of *E. coli* range from red to violet

(Stein and Bochner, 1998). For high background microflora populations that would be encountered in farm samples, the manufacturer recommends use of tellurite and novobiocin to increase selectivity of Rainbow® agar O157 (Stein and Bochner, 1998). Bettelheim (1998) concluded that the black color produced by EHEC strains is of assistance as the colonies provide a great contrast with the pink or violet colonies produced by non-EHEC strains. The author also noted that many of the non-sorbitol-fermenting non-*E. coli* isolated from SMAC would not grow on Rainbow® agar O157. Onoue et al. (1997) determined that Rainbow® agar O157 was inferior to CT-SMAC for recovery of *E. coli* O157:H7 following enrichment. Novicki et al. (1998) found that Rainbow® agar O157 was better at recovery of *E. coli* O157:H7 than SMAC from human stools. Taormina et al. (1998) found that colonies of heat stressed *E. coli* O157:H7 on Rainbow® agar O157 were not uniform in size or color.

Y. enterocolitica has been shown to grow well, although slowly, on non-selective media such as sheep blood agar, MacConkey agar, and Hektoen-Enteric agar (Bottone, 1999). The FDA Bacteriological Analytical Manual (FDA, 1995) recommends plating on MacConkey agar and cefsulodin-irgasan-novobiocin agar (CIN) for isolation of *Y. enterocolitica*. On MacConkey agar, the organism appears as flat, colorless, or pale pink colonies, 1-2 mm in diameter, after 24 hr incubation at 30°C (FDA, 1995). Colonies on CIN agar have a deep red center with a sharp border surrounded by a clear, colorless zone. The colonies have an entire edge, and are 1-2 mm in diameter after 24 hr incubation at 30°C (FDA, 1995). Bottone (1999) suggests that the colonial morphology of *Y. enterocolitica* results in difficulty in isolation from fecal specimens that contain large numbers of Enterobacteriaceae. Hamama et al. (1992) found that CIN agar was

more efficient at recovery of *Yersinia* spp. than MacConkey agar from various milk products. They found 33 of 227 samples positive for *Yersinia* using enrichment followed by plating to CIN, while only 13 samples were positive using MacConkey agar. Hamama et al. (1992) showed that other organisms that resembled *Yersinia* on MacConkey agar included *E. coli*, *Hafnia*, and *Serratia*. Davey et al. (1983) found that strains of *Proteus vulgaris*, *Proteus mirabilis*, and *Salmonella* spp. formed colonies similar to *Y. enterocolitica* on MacConkey agar. CIN agar was superior to MacConkey and cellibiose-arginine-lysine agars in the recovery of *Y. enterocolitica* or related species from cattle feces; ease of recognition and frequency of isolation of *Y. enterocolitica* were both factors in the conclusion (Davey et al., 1983). Using pure cultures, Davey et al. (1983) reported that CIN inhibited a majority of enteric organisms, although strains of *Citrobacter freundii*, *Serratia marcescens*, *Serratia* spp. and a strain of *Proteus vulgaris* formed colonies similar to *Y. enterocolitica* on the medium.

Another medium, KV-202 agar, has been identified as having similar recovery of *Y. enterocolitica* to CIN agar when using the same enrichment procedure in ground beef and ground pork (Jiang et al., 1998). The authors reported that KV-202 agar has an advantage over CIN agar in that it allows for differentiation of *Salmonella* and *Serratia* from *Y. enterocolitica*. The basis for detection of *Y. enterocolitica* on KV-202 agar is alkali production by the organism. The indicator dye in the medium is brilliant yellow, which changes from yellow to red at pH 8.0. In addition, failure to hydrolyze esculin is used to differentiate pathogenic *Y. enterocolitica* (does not hydrolyze esculin) from *Salmonella* spp., *Serratia* spp., and other *Yersinia* spp. (Vichienroj, 1997). Vichienroj (1997) found that inoculation of KV-202 and CIN agar with 10^8 CFU/ml of competitive

microflora (including *Serratia* and *Salmonella* spp.) resulted in growth of between 1 and 10 CFU/ml of competitive microflora on the media. The same study indicated an isolation rate of *Y. enterocolitica* of 32% for CIN and 30% for KV-202 agar after selective enrichment in sorbitol bile broth at 25˚C for 24 hr for ground pork samples. False positive detection rates were 46% and 16% for CIN and KV-202, respectively (Vichienroj, 1997).

Enrichment Methods

Current FDA BAM protocol for enrichment for *E. coli* O157:H7 includes modified tryptic soy broth (mTSB) and EHEC enrichment broth (EEB) (FDA, 1995). The enrichment broth described by the United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) for the detection of *E. coli* O157:H7 in meat and poultry products is modified *E. coli* broth with novobiocin (mEC+N; USDA, 1998). EEB and mTSB have the same base ingredients, including novobiocin, while EEB contains three other antimicrobials: cefixime, cefsulodin, and vancomycin. These substances make EEB highly selective for *E. coli* O157:H7. Modified *E. coli* broth is similar to mTSB, although lactose is the carbohydrate in mEC instead of glucose.

Heuvelink et al. (1997) found that EEB was inhibitory to *E. coli* O157 strains in pure culture studies. Weagant et al. (1995) found that enrichment in EEB followed by plating on CT-SMAC was superior to enrichment in mTSB followed by plating to HC for detecting *E. coli* O157:H7 in raw milk, sprouts, raw oysters, and raw hamburger. The same study revealed a sensitivity of one organism per 10 g of food sample using the EEB/CT-SMAC combination. Taormina et al. (1998) recommended that mTSB supplemented with novobiocin (mTSB+N) be used for detecting low numbers of heat-

stressed *E. coli* O157:H7 in ground beef. Feldsine et al. (1997) concluded that mTSB+N did not allow enough specificity in favoring EHEC organisms over other Gram-negative bacteria in various food samples. Restaino et al. (1996) found that mEC+N detected of *E. coli* O157:H7 in ground beef patties at populations as low as 0.1 CFU/g of sample after 24 hr of incubation. Onoue et al. (1997) found that mEC+N followed by plating to CT-SMAC was nearly as effective as immunomagnetic separation. Blais et al. (1997) reported that mEC+N supported growth of *E. coli* O157:H7 best in pure culture and in ground meat when incubated statically at 42˚C. They suggested that injury may have occurred and that enrichment at 42˚C is either more effective than enrichment at 37˚C for recovery or less inhibitory. Sata et al. (1999) found that mEC+N and mTSB+N suppressed growth of starved *E. coli* O157:H7 when compared to non-selective enrichment media such as TSB.

For recovery of *Y. enterocolitica* from foods, FDA describes cold enrichment (10˚C, 10 d) in peptone sorbitol bile broth (FDA, 1995). Miller et al. (1997) found 0% incidence of *Y. enterocolitica* in pork carcasses using this methodology, while VanNoyen et al. (1980) found that cold enrichment recovered mostly non-pathogenic isolates of *Y. enterocolitica* from feces of infected humans. Shiemann and Olson (1984) reported that use of temperatures above 15˚C decreases the competitive ability of *Yersinia* in relation to other Gram-negative bacteria.

Rapid Methods

Immunomagnetic separation (IMS) followed by plating on selective media has been used to increase detection of *E. coli* O157:H7. IMS is accomplished by first covalently bonding *E. coli* O157 antibodies to polystyrene beads that contain a magnetic

component. The beads bind to target bacteria (via antigen-antibody interaction) in an enrichment broth and are separated from the sample matrix using a magnet. Chapman et al. (1994) found that IMS followed by plating to CT-SMAC or CR-SMAC was 100-fold more sensitive than direct culture on these media for recovery of most strains of *E. coli* O157:H7 in bovine feces. Porter et al. (1997) found that IMS was possible in such samples as water, feces, fecal slurry, grass, and soil. The study also indicated that a strain of *Enterobacter agglomerans* bound to the magnetic beads at high rates and had similar colony morphology to *E. coli* O157 on SMAC supplemented with MUG. Karch et al. (1996) showed that IMS followed by direct plating on CT-SMAC detected 10^2 CFU of *E. coli* O157/g of human stool. Weagant et al. (1995) found that IMS was slightly less sensitive than enrichment in EEB followed by plating to CT-SMAC for recovery of *E. coli* O157:H7 from various food samples. IMS was found to be more sensitive than direct plating for detection of *E. coli* O157:H7 in a survey of cattle fecal samples (Van Donkersgoed, 1999).

Enzyme-linked immunosorbent assays (ELISA) have been employed to detect *E. coli* O157 from various samples. ELISA techniques involve detection of the O157 antigen in enrichment broths. EHEC-Tek® (Organon-Teknika) is a commercially available ELISA, and the protocol has been modified to utilize IMS beads to increase specificity (Johnson et al., 1995). Johnson et al. (1995) found that incorporating acriflavine into a secondary enrichment increased the ELISA reactivity. The same study reported that inoculum levels as low as 0.1 CFU/g could be detected in raw ground beef using the modified protocol. Using EHEC-Tek®, Restiano et al. (1996) obtained 100% recovery of *E. coli* O157:H7 in artificially inoculated ground beef with levels ranging

from 0.1 to 100 CFU/g. Chapman and Siddons (1996) found that of 108 beef samples inoculated with *E. coli* O157:H7 at levels of 2.0 to 0.02 CFU/g, 98 were positive by EHEC-Tek®. The investigators showed that buffered peptone water supplemented with vancomycin, cefsulodin, and cefixime (BPW-VCC) improved the performance of the assay over the manufacturer's recommended enrichment medium, mEC+N.

Another assay currently used for the detection of *E. coli* O157 is the Reveal® for *E. coli* O157:H7 test system. This device relies on a antigenic detection of the organism, which can be easily viewed as a line formed in the viewing port in the test device. The device can detect as few as 10^4 CFU of *E. coli* O157:H7/ml of enrichment broth. The device employs two enrichment options, a proprietary medium (8 hr enrichment), or mEC+N (20 hr enrichment) prior to inoculation in the test device (Neogen, 1998).

OBJECTIVES OF CURRENT RESEARCH

Recent research in food microbiology has established an undeniable link between bacteria from farm environments and contamination of foods. In many cases, farm components (e.g., feed) can be vehicles for transmission of infectious organisms to the food producing animal. Therefore, increased importance has been placed on detection of the organism in these products before the animal enters the food cycle, thereby decreasing the likelihood of contamination of the finished product.

E. coli O157:H7 and *Y. enterocolitica* are two pathogenic bacteria that are detected from farm environments with frequency. The purpose of this research was to determine if current techniques for detection of these microorganisms in food are applicable to farm environments as well. The farm environment presents an increased

challenge in the ultimate goal of reliable detection of pathogenic microorganisms. Therefore, we must evaluate current methodology to establish whether our current protocols are suitable for such an undertaking.

The objectives for the detection of *E. coli* O157:H7 in farm environments were: (1) to establish whether direct plating on selective/differential media can recover high levels of the organism; (2) establish whether current enrichment techniques, designed for recovery from food products, followed by plating on selective/differential media can recover low levels of the organism; and (3) establish whether rapid methods currently in use in the food industry are suitable for detection of low levels of the organism in the farm environment.

The objectives for the detection of *Y. enterocolitica* in various farm environments were to determine if direct plating on selective/differential media can recover high levels of the organism, and to establish whether current enrichment techniques followed by plating on selective/differential media can recover low levels of the organism.

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Part II:

Detection and Enumeration of *Escherichia coli* O157:H7 from Farm Animal Environments

ABSTRACT

Recovery of *Escherichia coli* O157:H7 from farm samples (feed, bedding, fecal samples, and water) from various animal sources (cattle, swine, and chickens) was investigated at inoculum levels of 10^4 CFU/g (high level) and 10 CFU/g (low level) of sample. High-inoculum levels of *E. coli* O157:H7 were recovered from samples using direct plating on sorbitol MacConkey agar (SMAC), SMAC supplemented with cefixime and tellurite (CT-SMAC), hemorrhagic colitis agar (HC), modified eosin methylene blue agar (MEMB), and Rainbow® agar O157. Low-inoculum levels of *E. coli* O157:H7 were recovered using a 24-hr enrichment at 37˚C in modified tryptic soy broth with novobiocin (mTSB+N), EHEC enrichment broth (EEB), and modified *E. coli* broth with novobiocin (mEC+N) followed by streaking onto selective media. In addition, low-inoculum levels of *E. coli* O157:H7 were compared for detection using immunomagnetic separation (IMS), EHEC-Tek®, and Reveal® for *E. coli* O157:H7 test systems. Eight sample types were evaluated. For direct plating, three repetitions performed, giving a total of 24 (8x3) inoculated samples tested. Duplicate sample types were examined for low inoculum evaluations, also performed in triplicate, for a total of 48 (8x2x3) individual samples evaluated. Rainbow® agar O157 recovered high-inoculum levels of *E. coli* O157:H7 by direct plating from the most sample types (7 of 8), but provided the poorest numerical recovery in 6 of 7 sample types from which the organism was recovered ($P<0.05$). CT-SMAC and Rainbow® agar O157 recovered *E. coli* O157:H7 from 18 of 24 individual samples. CT-SMAC and MEMB recovered *E. coli* O157:H7 by direct plating from 6 of 8 sample types. MEMB provided the best numerical recovery in total mixed ration cattle feed (TMR), cow fecal, and pig feed sample types ($P<0.05$).

Using enrichment, mTSB+N followed by streaking onto CT-SMAC provided best recovery (18 of 48 samples tested). The EHEC-Tek® test system provided positive results in 18 of 24 inoculated samples, with chicken fecal/bedding mixture not providing a positive result (0 of 3). The Reveal® for *E. coli* O157:H7 test system provided positive results in 20 of 24 inoculated samples. IMS performed best when preparations were plated onto Rainbow® agar O157 (10 of 24 positive), but the method was inferior to EHEC-Tek® and Reveal® *E. coli* O157:H7 for recovery of low-inoculum levels of the organism ($P<0.05$).

INTRODUCTION

Escherichia coli O157:H7 has been shown to survive well in the diverse niches that exist in farm animal environments. These niches must be investigated to determine the potential for contamination of animals or their products used as food. There is little done in the United States to monitor the bacterial quality of animal feeds (Hancock et al., 1997). Feeds have been shown to harbor high levels of *E. coli*, and while *E. coli* O157 was not isolated, the organism has been shown to survive in inoculated feed samples (Lynn et al., 1998). *E. coli* O157 has been found in cattle feces at frequencies ranging from 1.4% to 15.7% (Bonardi et al., 1999; Chapman et al., 1997; Hancock et al., 1997a, 1997b). While *E. coli* O157 has been isolated from pig feces, these strains were unlikely as a source of human infection (Chapman et al., 1997). It has been shown that chickens infected with *E. coli* O157:H7 can shed the organism for up to 90 days (Beery et al., 1985). Water has been shown to be a vector of *E. coli* O157:H7 in several circumstances (Geilreich et al., 1992; MMWR, 1996; Warner et al., 1996), and the organism has been shown to survive for >300 days in sterilized bottled water (Warburton et al., 1998).

Plating media used for detection of *E. coli* O157:H7 have commonly exploited the lack of sorbitol fermentation by *E. coli* O157:H7 as compared to *E. coli* and other enteric organisms that do ferment sorbitol. Sorbitol MacConkey agar (SMAC) is used to differentiate *E. coli* O157:H7 from non-pathogenic *E. coli* on the basis of sorbitol utilization (March and Ratnam, 1986), and the medium has been effective for isolating the organism from human feces (Krishnan et al., 1987). However, questions about effective recovery of *E. coli* O157:H7 with SMAC from other environments with high numbers of background microflora have arisen (Padhye and Doyle, 1991). The addition

of cefixime and tellurite to SMAC (CT-SMAC) has increased the selectivity of the medium by inhibiting several other sorbitol non-fermenting bacteria (Weagant et al., 1995; Zadik et al., 1993). CT-SMAC has been shown to have greater recovery of *E. coli* O157:H7 in minced beef than SMAC (Onoue et al., 1997). Hemorrhagic colitis agar (HC) has been used to detect *E. coli* O157:H7 on the basis of no sorbitol fermentation and inability to hydrolyze 4 methyl-umbelliferyl-*beta*-D-gluconuride (MUG) and produce fluorescence. Unlike 94% of *E. coli*, *E. coli* O157:H7 does not produce the enzyme *beta*-glucuronidase, responsible for hydrolyzing MUG and producing 4-methylumbelliferone and fluorescence upon exposure to UV light (FDA, 1995). This medium has been shown to recover heat stressed *E. coli* O157:H7 from various dairy products as well as SMAC. HC was superior to SMAC for recovery of unstressed cells from cheese (Hammack et al., 1997). However, HC has been criticized for the nondistinctive characteristics of *E. coli* O157:H7 colonies on the medium, thus requiring many colonies be picked for confirmation (Feldsine et al., 1997a, 1997b). Modified eosin methylene blue agar (MEMB) also exploits the inability of *E. coli* O157:H7 to ferment sorbitol, and has been shown to have greater recovery in meat products than SMAC (Clavero and Beuchat, 1995; Taormina et al., 1998). Rainbow® agar O157 is a relatively new medium that relies on the breakdown of chromogenic substrates to differentiate *E. coli* O157:H7 from other *E. coli* (Stein and Bochner, 1998). Selectivity of Rainbow® agar O157 with high background microflora samples, such as cattle feces, is increased by addition of novobiocin and tellurite (Stein and Bochner, 1998)

Currently the Food and Drug Administration's Bacteriological Analytical Manual (BAM) lists two enrichment broths for detection of *E. coli* O157:H7 in foods: modified

tryptic soy broth with novobiocin (mTSB+N) and EHEC enrichment broth (EEB) (FDA, 1995). The United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) describes using modified *E. coli* broth with novobiocin (mEC+N) to recover *E. coli* O157:H7 from meat and poultry products (USDA, 1998). EEB has been found to be inhibitory to *E. coli* O157 strains in pure culture studies (Heuvelink et al., 1997), but has been used to isolate the organism from unprocessed food products (Weagant et al., 1995). Low numbers of heat-stressed *E. coli* O157:H7 have been recovered from ground beef using mTSB+N (Taormina et al., 1998), but the medium has been shown not provide enough suppression of background microflora to recover EHEC organisms (Feldsine et al., 1997a, 1997b) as well as inhibiting growth of starved *E. coli* O157:H7 (Sata et al., 1997). Recovery of as few as 0.1 CFU/g of *E. coli* O157:H7 has been achieved from ground meat using mEC+N (Blais et al., 1997; Restaino et al., 1996).

Techniques have been developed to improve specificity and decrease detection time for *E. coli* O157:H7. These include immunomagnetic separation (IMS), enzyme-linked immunosorbent assays (ELISA), and other antibody-based systems. IMS is more sensitive than direct plating for detecting *E. coli* O157:H7 in bovine feces (Chapman et al., 1994; Van Donkersgoed et al., 1999), and has detected *E. coli* O157 concentrations as few as 10^2 CFU/g human stool in the presence of 10^7 coliform background flora (Karch et al., 1996). EHEC-Tek® (Organon Teknika, Durham, NC), an ELISA based test system, also incorporates IMS to improve specificity. EHEC-Tek® with IMS has been shown to detect *E. coli* O157:H7 at levels as low as 0.1 CFU/g in artificially inoculated ground beef (Johnson et al., 1995; Restaino et al., 1996). The Reveal® for *E. coli* O157:H7 test device incorporates *E. coli* O157:H7 antibodies for detection, as indicated by a colored

antigen/antibody band and requires only 10^4 CFU *E. coli* O157:H7/ml following enrichment.

While most improvements in detection methods for *E. coli* O157:H7 have focused on recovery from foods, little effort has been made to evaluate these methods with non-food samples heavily contaminated with competitive background microflora. Therefore, the objective of this investigation was to evaluate various direct-plating, enrichment, and rapid-test protocols commonly used for testing foods for their efficacy for recovery of *E. coli* O157:H7 from various farm animal environments.

MATERIALS AND METHODS

Samples Evaluated

Farm animal sample types evaluated were as follows: (1) Water - obtained from dairy cattle ball drinkers containing water from the Knox County municipal water system, (2) Total mixed ration dairy cattle feed (TMR) - contained corn silage, rye silage, alfalfa hay, rye hay, and protein concentrate, obtained from areas where cows had made contact with the feed, (3) Cow fecal material - obtained from floor of dairy cattle holding pens; a mixture of feces, urine, and other matter, (4) Pig fecal material - obtained from floor of holding pens, a mixture of feces, urine, water, and other matter, (5) Chicken feed - 16% complete crumble containing 16% crude protein, 0.67% lysine, 0.33% methionine, 3% crude fat, 5% crude fiber, 4.25% calcium, 0.5% phosphorous, and 0.7% salt, obtained from chicken feeders where birds made contact with feed, (6) Chicken fecal material - contained a mixture of sawdust, feces, feathers, and other matter obtained from floor of the holding pens, (7) Pig feed - Contained corn, soybean meal, crude protein, crude fiber,

crude fat, calcium, phosphorus, other vitamins and minerals, and oxytetracycline and tylosin phosphate antibiotic supplements, (8) Bedding - Sawdust material obtained from dairy cattle stalls.

All samples were obtained from University of Tennessee Agricultural Experiment Station farms in Knoxville, TN. After collection, samples were immediately transported on ice to the laboratory, and held at 4°C until analyzed (≤ 7 days). Samples were removed from the refrigerator and allowed to warm to ambient temperature (approximately 20°C) prior to analysis.

Inoculum Preparation

Five strains of *E. coli* O157:H7 were used in this study: a cider isolate and ground beef isolate obtained from M.P. Doyle, University of Georgia, Griffin, GA; a salami isolate, obtained from A.M. McNamara, USDA-FSIS, Washington DC; and ATCC strains 43889 and 43894 obtained from American Type Culture Collection, Rockville, MD. Strains were grown in tryptic soy broth (TSB) at 37°C and subjected to a minimum of two successive 24-hr transfers prior to inoculation into samples. Two milliters of each test strain were combined in a sterile centrifuge tube resulting in a mixed culture containing equal proportions of the five strains. The suspension was centrifuged at 11,068 x *g* for 10 min at 4°C. The supernatant was decanted, cells were resuspended in 10 ml of 0.1% peptone water (PW), and the process was repeated. The population of the mixed culture (10^9 CFU/ml) was determined by surface plating onto tryptic soy agar and incubation at 37°C for 24 hr.

Direct Plating

Media Preparation

Five media were evaluated for their ability to recover *E. coli* O157:H7 from farm samples. These include sorbitol MacConkey agar (SMAC), SMAC supplemented with cefixime and tellurite (CT-SMAC), hemorrhagic colitis agar (HC), modified eosin methylene blue agar (MEMB) and Rainbow® agar O157. All media were prepared as detailed in Appendix I.

Inoculation of Samples/Direct Plating Procedure

Ten grams or milliters of test samples were placed in sterile stomacher bags fitted with nylon mesh filters (Nasco). The mixed culture was serially diluted in PW to 10^5 CFU/ml and 1 ml of the 10^5 CFU/ml suspension was inoculated into samples to provide an inoculum of about 10^4 CFU/g or ml of sample. Samples were mixed by hand to distribute the inoculum throughout the sample. For control samples, 1 ml of PW was added. Inoculated samples or controls were held for 1 hr at ambient temperature (21-24°C). After 1 hr, 10 ml of PW were added to samples which were pummeled for 2 min in a stomacher lab blender. The resulting suspension was diluted as appropriate in PW and surface plated (0.1 ml) onto duplicate agar test media. Media were incubated for 18-48 hr at 37°C, before typical *E. coli* O157:H7 colonies were enumerated.

Confirmation Procedure

Typical *E. coli* O157:H7 colonies (up to five) from test media were streaked onto tryptic soy agar containing yeast extract (TSAYE). The isolates were incubated for 24 hr at 37°C before confirmation tests were performed. Isolates were screened using the spot indole test, with indole-positive isolates being further evaluated for Gram reaction, methyl red, Voges-Proskauer, citrate utilization (Koser's formulation), production of gas from lactose (lauryl tryptose broth), and *E. coli* O157 antigen latex agglutination (Oxoid,

Ogdensburg, NY). All Gram-negative, indole-positive, methyl red-positive, Voges-Proskauer-negative, citrate-negative, gas producers from lactose, and serologically positive isolates were identified as *E. coli* O157:H7.

Counts obtained from direct plating studies reflect confirmed positive isolates and were transformed to log CFU/g or ml of sample. Confirmed counts were obtained using the following formula: (number of confirmed positive isolates/number of isolates tested) x number of colonies counted. For example, for 100 colonies counted with five isolates selected for confirmation and four confirmed positive, the reported count would be: (4/5) x 100 = 80 CFU/g = 1.90 log CFU/g.

Enrichment Methods

Media Preparation

Three enrichment broths, EHEC enrichment broth (EEB), modified tryptic soy broth with novobiocin (mTSB+N), and modified *E. coli* enrichment broth (mEC+N) were evaluated in conjunction with five plating media (SMAC, CT-SMAC, HC, MEMB, and Rainbow® agar O157). Media were prepared as described in Appendix I.

Inoculation of Samples/Enrichment Procedure

Test samples (25 g or ml) were added to sterile stomacher bags fitted with nylon mesh filters (Nasco). The 24-hr *E. coli* O157:H7 mixed culture was diluted to 10^2 CFU/ml in PW, and 2.5 ml of this dilution were inoculated into test samples to provide an inoculum of about 10 CFU/g or ml of sample. Control samples, were inoculated with 2.5 ml of PW. Samples were held for 1 hr under ambient temperatures (21-24°C). After 1 hr, 225 ml of appropriate enrichment broth was added to samples followed by pummeling for 2 min in a stomacher blender. Enrichment suspensions were incubated at

37°C with shaking at 100 rpm for 24 hr. After 24 hr, the sample was removed from the incubator and suspensions were streaked onto SMAC, CT-SMAC, HC, MEMB, and Rainbow® agar O157. Media were incubated for 18-48 hr at 37°C, and typical *E. coli* O157:H7 colonies were confirmed as described previously.

Rapid Methods

Sample Preparation

Rapid test protocols describe here utilized the incubated enrichment samples described above.

*Dynabeads® anti-*E. coli O157

For this evaluation, the mTSB+N enrichments were used. After incubation for 24 hr at 37°C as previously described, 1 ml was taken from each enriched sample for analysis, with the exception of pig and cow fecal samples. For extremely particulate samples, as were pig and cow fecal samples, the manufacturer recommends using a two-fold dilution of enriched samples. Therefore, 0.5 ml of enrichment broth from these two samples were diluted with 0.5 ml of phosphate buffered saline with Tween 20 (PBS-Tween; Sigma) in 1.5 ml microcentrifuge tubes containing 20 µl of suspended Dynabeads® anti-*E. coli* O157. The tubes were placed in a sample mixer (Dynal MX3; Dynal, Lake Success, NY) and agitated for 10 min at ambient temperature. The microcentrifuge tubes were then placed in a magnetic particle concentrator (Dynal MPC®-M; Dynal, Lake Success, NY),and beads were allowed to concentrate for 3 min, after which time the supernatant was aspirated with the magnet in place. One ml of PBS-Tween was added to tubes, the magnet was removed, and the bead pellet was resuspended. After two more separations and washes, the pellet was resuspended in 100

µl PBS-Tween and mixed gently on a vortex to resuspend the beads. The bead-bacteria complexes were streaked onto SMAC, CT-SMAC, HC, MEMB, and Rainbow® agar O157 and plates were incubated at 18-48 hr at 37°C. Typical *E. coli* O157:H7 colonies were confirmed as described previously.

EHEC-TEK® Test System

The EHEC-TEK® test system (Organon Teknika, Durham, NC) incorporates Dynal Dynabeads® into the protocol of an ELISA (enzyme-linked immunosorbent assay) for the detection of *E. coli* O157:H7. The Dynabeads® protocol used for the EHEC-Tek® is similar to the one listed previously, with the exception that mEC+N enrichment samples incubated for only 6 hr were used. In addition, the bead-bacteria complexes were suspended in a secondary enrichment of TSB supplemented with 0.01% (w/v) acriflavine in place of suspension in 100 µl of PBS-Tween. The secondary enrichment was incubated for 18 hr at 37°C, after which time it was placed in a boiling water bath for 20 min. The boiled sample was stored at 4°C for up to 72 hr, as recommended by the manufacturer. The prepared sample was warmed or cooled to ambient temperature, and the ELISA portion of the test was performed as directed by the manufacturer. The ELISA plates were read using an OTC 400 microplate reader (Organon Teknika, Durham, NC). Samples were considered positive if the reading was greater than or equal to 0.250 + the average of the negative control values.

Reveal® for E. coli O157:H7 *Test System*

For the Reveal® for *E. coli* O157:H7 test 20 hr enrichments in mEC+N were used. All test devices were stored at 4°C, but were warmed to room temperature (ca. 22°C) before use. The filter bag was shaken in a side-to-side motion for 5 sec, and a 120 µl

sample of enrichment broth was removed. The sample was inoculated into the portal of the test device. The presence of color-indicated lines in both the C and T zones of the device within 15 min was indicative of a positive test.

Measurement of pH

Samples were evaluated for pH using a modified AOAC method for food, placing 10 g of sample in 100 ml of distilled and reading pH after 1 hr. In addition, samples were evaluated in peptone water (90 ml in 10 g or ml), and mTSB, EEB, and mEC enrichment broths (225 ml in 25 g or ml). Diluent/Enrichment samples were evaluated after inoculation with PW and allowed to rest 1 hr.

Statistical Analysis

All sample types were analyzed by test methods in triplicate. Recovery of *E. coli* O157:H7 by direct plating was statistically analyzed using the mixed procedure (PROC MIXED) of SAS version 7.00 (SAS Institute, Cary, NC). The model was a randomized block design with replication, blocked on replication. Means were separated using LSMeans.

RESULTS

The pH measurements for various farm samples are shown in Table 1. Samples ranged in pH from 5.18 (TMR) to 8.02 (cow feces) using the modified AOAC method. The pH within enrichment broths varied slightly, depending on sample type.

Recovery and enumeration data for high levels *E. coli* O157:H7 by direct plating are shown in Table 2. For the purpose of this study, recovery refers to the ability to

Table 1. The pH of various farm samples measured in diluent or enrichment broths.

Sample	Diluent/Enrichment Broths			
	Distilled Water	0.1% Peptone Water	mTSB/EEB[1]	mEC
Water	7.69	7.91	7.36	7.12
TMR	5.18	5.16	7.02	6.99
Cow Fecal	8.02	8.20	7.42	7.23
Pig Fecal	7.25	7.30	7.24	7.14
Chicken Feed	6.56	6.61	7.19	7.08
Chicken Fecal	7.00	8.06	7.33	7.16
Pig Feed	6.27	6.40	7.16	7.10
Bedding	7.38	7.81	7.34	7.15

[1] Means were the same and reported as one value

Table 2. Numerical recovery of high-inoculum level (~log 4 CFU/g or ml) of *E. coli* O157:H7 from farm samples[1] by direct plating onto various selective media.

Sample	Population recovered (log CFU/g or ml) on[2]:				
	SMAC	CT-SMAC	HC	MEMB	Rainbow® agar O157
Water	4.00 ab	4.11 a	4.21 a	3.70 b	2.80 c
TMR	3.93 b	3.84 bc	ND[3] d	4.51 a	3.59 c
Cow Fecal	ND c	ND c	ND c	4.38(1) a	3.50 b
Pig Fecal	3.92(2) ab	3.56 b	4.22(1) a	4.11(2) a	3.25(2) c
Chicken Feed	3.88 a	3.84 a	3.91 a	3.87 a	3.41 b
Chicken Fecal	ND c	3.87 a	ND c	ND c	3.30(1) b
Pig Feed	3.98 ab	3.53 c	3.87(2) b	4.23 a	3.17 d
Bedding	ND	ND	ND	ND	ND

[1] *E. coli* O157:H7 was not recovered from the controls.

[2] Data are means of 3 replications unless otherwise noted in parentheses. Means for less than 3 replications were used when the organism was not detected in one or more replications. Means in the same row followed by different letters are significantly different ($P<0.05$).

[3] Not detected; detection was not permissible due to growth of background microflora (not *E. coli* O157:H7) at levels exceeding the inoculation population of *E. coli* O157:H7.

simply identify and confirm the presence of *E. coli* O157:H7 colonies on selective media, regardless of the accuracy of enumerating the organism. Numerical recovery refers to the efficacy at which the media allowed for enumeration of known, inoculated populations of *E. coli* O157:H7. Overall, no medium could be identified as superior to others for all sample types, with variable differences in recovery and numerical recovery being observed. No medium was suitable for recovery of the organism from bedding due to overgrowth by indigenous microflora. Rainbow® agar O157 provided the lowest numerical recovery of *E. coli* O157:H7 in all samples, and it was poorer than other test media for numerical recovery from chicken feed, pig feed, and water samples ($P<0.05$). Rainbow® agar O157 and CT-SMAC recovered *E. coli* O157:H7 from the most farm samples (18 of 24), although CT-SMAC was not able to recover the organism from the cow fecal sample. CT-SMAC provided the best numerical recovery in the chicken fecal sample ($P<0.05$). MEMB provided the best numerical recovery from the TMR and cow fecal sample, although the medium was only provided recovery from 1 of 3 cow fecal samples ($P<0.05$). MEMB was not able to recover the organism from chicken fecal and bedding sample types. Overall, MEMB recovered *E. coli* O157:H7 from 15 of 24 farm samples. For water, pig fecal, chicken feed, and pig feed sample types, recovery on SMAC was among the best although not significantly different from some other media ($P<0.05$). However, recovery of *E. coli* O157:H7 from cow fecal, chicken fecal, and bedding sample types was not obtained with SMAC. Overall, SMAC recovered *E. coli* O157:H7 from 13 of 24 farm samples. Overall, HC provided recovery from 9 of 24 farm samples, but was not significantly different than some of the other media for recovery from water, pig fecal, and chicken feed sample types ($P<0.05$). HC was inferior to

MEMB in recovery from pig feed sample type ($P<0.05$), and did not provide recovery from TMR, cow fecal, chicken fecal, and bedding sample types.

Recovery data for low levels of *E. coli* O157:H7 by enrichment followed by streaking onto selective media is shown in Table 3. The organism was not recovered by any enrichment method from the bedding sample. Across all samples and media types, mEC+N enrichment provided the greatest recovery (35 of 120 samples; 29%) followed by mTSB+N (58 of 240 samples; 24%), and EEB (42 of 240 samples; 18%). The most effective enrichment-selective media combinations across all sample types were as follows: mTSB+N/CT-SMAC (18 of 48; 38%), mEC+N/SMAC (8 of 24; 33%), mEC+N/CT-SMAC (7 of 24; 29%), mEC+N/HC (7 of 24; 29%), and mEC+N/Rainbow® agar O157 (7 of 24; 29%). For the TMR sample mTSB+N/CT-SMAC (6 of 6), mEC+N/CT-SMAC (3 of 3), mEC+N/SMAC (3 of 3), and mEC+N/HC (3 of 3) combinations provided the greatest recovery. The organism was recovered in the chicken fecal sample using mTSB+N/HC (1 of 6) and mTSB/Rainbow® agar O157 (1 of 6). For the chicken feed sample, mTSB+N/CT-SMAC (6 of 6), mTSB+N/Rainbow® agar O157(6 of 6), EEB/CT-SMAC (6 of 6), and mEC+N (3 of 3) provided the greatest recovery. The organism was recovered from 1 of 6 samples using mTSB+N/CT-SMAC, EEB/Rainbow® agar O157, and EEB/CT-SMAC combinations in the pig feed sample. The mTSB+N/MEMB combination (2 of 6) provided the greatest recovery from the pig fecal sample. From the water sample, mEC+N/MEMB (3 of 3) and mEC+N/Rainbow® agar O157 (3 of 3) provided the greatest recovery. The organism was recovered by mTSB+N/Rainbow® agar O157 (1 of 6), mTSB+N/CT-SMAC (1 of 6), and EEB/HC (1 of 6) in the cow fecal sample.

Table 3. Detection of low-inoculum levels (~10 CFU/g or ml) of *E. coli* O157:H7 from farm samples[1] using various enrichments followed by streaking onto selective media.

Sample	Selective Medium	Enrichment Medium		
		mTSB+N	EEB	mEC+N
Water	SMAC	1/6[2]	1/6	2/3
	CT-SMAC	4/6	0/6	2/3
	HC	0/6	0/6	2/3
	MEMB	1/6	0/6	3/3
	Rainbow® agar O157	2/6	0/6	3/3
TMR	SMAC	4/6	4/6	3/3
	CT-SMAC	6/6	3/6	3/3
	HC	4/6	3/6	3/3
	MEMB	3/6	3/6	2/3
	Rainbow® agar O157	3/6	2/6	2/3
Cow Fecal	SMAC	0/6	0/6	0/3
	CT-SMAC	1/6	0/6	0/3
	HC	0/6	1/6	0/3
	MEMB	0/6	0/6	0/3
	Rainbow® agar O157	1/6	0/6	0/3
Pig Fecal	SMAC	0/6	0/6	0/3
	CT-SMAC	0/6	0/6	0/3
	HC	0/6	0/6	0/3
	MEMB	2/6	1/6	0/3
	Rainbow® agar O157	0/6	0/6	0/3

-continued-

Table 3, continued.

Sample	Selective Medium	Enrichment Medium		
		mTSB+N	EEB	mEC+N
Chicken Feed	SMAC	3/6	4/6	3/3
	CT-SMAC	6/6	6/6	2/3
	HC	4/6	4/6	2/3
	MEMB	4/6	3/6	1/3
	Rainbow® agar O157	6/6	5/6	2/3
Chicken Fecal	SMAC	0/6	0/6	0/3
	CT-SMAC	0/6	0/6	0/3
	HC	1/6	0/6	0/3
	MEMB	0/6	0/6	0/3
	Rainbow® agar O157	1/6	0/6	0/3
Pig Feed	SMAC	0/6	0/6	0/3
	CT-SMAC	1/6	1/6	0/3
	HC	0/6	0/6	0/3
	MEMB	0/6	0/6	0/3
	Rainbow® agar O157	0/6	1/6	0/3
Bedding	SMAC	0/6	0/6	0/3
	CT-SMAC	0/6	0/6	0/3
	HC	0/6	0/6	0/3
	MEMB	0/6	0/6	0/3
	Rainbow® agar O157	0/6	0/6	0/3

[1] *E. coli* O157:H7 was not recovered from the controls.
[2] Values indicate number of confirmed positives/total number of observations.

Results for recovery of low inoculum levels using IMS (Dynabeads®), EHEC-Tek®, and Reveal® for *E. coli* O157:H7 test systems are shown in Tables 4 and 5. Reveal® for *E. coli* O157:H7 provided positive results in 20 of 24 inoculated samples, but did not allow for detection in the pig feed sample. EHEC-Tek® recovered the organism from 18 of 24 samples. EHEC-Tek® did not detect the organism in the chicken fecal sample type. IMS (Dynabeads®) provided the greatest recovery across all samples when plated onto Rainbow® agar O157 (10 of 24), followed by plating to HC and SMAC (9 of 24), and CT-SMAC, MEMB, and SMAC (7 of 24).

DISCUSSION

Direct plating methods proved more effective for recovery when media were made more selective by incorporation of additional selective agents (e.g., cefixime and tellurite). However, poor numerical recovery was often a trade-off for better detection. Rainbow® agar O157 proved most effective at recovery across all sample types, recovering *E. coli* O157:H7 from all sample types except bedding. This may be attributed to the addition of novobiocin and tellurite to the medium, and the ease of recognition of black colonies on an opaque background. Rainbow® agar O157 allowed for ease at discerning *E. coli* O157:H7 in the cow fecal sample, thereby providing consistency of recovery (3 of 3 samples isolated). However, the medium was poorer at numerical recovery of *E. coli* O157:H7 than other media within sample types ($P<0.05$). Poor numerical recovery on Rainbow® agar O157, as well as other media, is attributed to two major factors. First, the addition of highly selective agents indeed reduces growth of competitive background microflora, but also is somewhat inhibitory to *E. coli* O157:H7,

Table 4. Detection of low inoculum levels (~10 CFU/g or ml) of *E. coli* O157:H7 from farm samples[1] after enrichment in mTSB+N, immunomagnetic separation (Dynabeads®), and streaking onto selective media.

Sample	Selective Medium				
	SMAC	CT-SMAC	MEMB	HC	Rainbow® agar O157
Water	2/3[2]	2/3	3/3	3/3	3/3
TMR	3/3	3/3	2/3	3/3	2/3
Cow Fecal	0/3	0/3	1/3	0/3	0/3
Pig Fecal	0/3	0/3	0/3	1/3	0/3
Chicken Feed	3/3	2/3	1/3	2/3	2/3
Chicken Fecal	0/3	0/3	0/3	0/3	2/3
Pig Feed	0/3	0/3	0/3	0/3	0/3
Bedding	1/3	0/3	0/3	0/3	1/3

[1] *E. coli* O157:H7 was not recovered from the controls

[2] Values indicate number of confirmed positives/total number of observations.

Table 5. Detection of low-inoculum levels[1] (~10 CFU/g or ml) of *E. coli* O157:H7 using EHEC-Tek® and Reveal® for *E. coli* O157:H7 test systems after enrichment in mEC+N.

Sample	EHEC-Tek®	Reveal® for *E. coli* O157:H7
Water	3/3[2]	3/3
TMR	3/3	3/3
Cow Fecal	3/3	3/3
Pig Fecal	2/3	3/3
Chicken Feed	3/3	3/3
Chicken Fecal	0/3	2/3
Pig Feed	1/3	0/3
Bedding	3/3	3/3

[1] *E. coli* O157:H7 was not recovered from the controls

[2] Values indicate number of confirmed positives/total number of observations.

especially if the cells have undergone stress. Second, poor numerical recovery may also be attributed to the inability to count and confirm *E. coli* O157:H7 colonies because of overgrowth of background microflora. CT-SMAC recovered *E. coli* O157:H7 from the same number of total samples as Rainbow® agar O157 (18). However, the medium was not able to recover *E. coli* O157:H7 from the cow fecal sample type due to overgrowth of background microflora (not *E. coli* O157:H7). Zadik et al. (1993) reported that suppression of non-O157 *E. coli* from cattle rectal swabs on CT-SMAC increased the number of non-sorbitol fermenting colonies (i.e., *E. coli* O157:H7), thereby demonstrating the importance of controlling growth of background microflora. In the present study, CT-SMAC provided better recovery than Rainbow® agar O157 in 5 of 6 samples from which both media recovered the organism ($P<0.05$). *E. coli* O157:H7 was not recovered from as many total samples using MEMB, but the medium was consistently the most effective at numerical recovery from samples in which the organism was recovered ($P<0.05$). For water samples, MEMB was inferior to SMAC and HC ($P<0.05$). SMAC and HC agars provided good numerical recovery from samples with low background microflora, although both performed poorly in cow fecal and chicken fecal samples. HC agar, which is not highly selective, was the only medium not able to recover *E. coli* O157:H7 from TMR. TMR has been shown to harbor populations of *E. coli* as high as 4.7 $\log_{10}$ CFU/g (Lynn et al., 1998), which could adversely affect recovery of *E. coli* O157:H7.

TMR, chicken feed, and water samples allowed for the greatest recovery by enrichment, accounting for 92% of positive samples (124 of 135). EEB was the least effective at recovery in all samples, which is substantiated by Heuvelink et al. (1997)

who reported that EEB was inhibitory to *E. coli* O157:H7 strains. The less selective mEC+N and mTSB+N support higher isolation rates. Blais et al. (1997) suggested that incubation of mEC+N at 42˚C is more effective at suppressing background microflora than incubation at 37˚C, which was utilized in the current study. The most effective enrichment/media combination proved to be a less selective enrichment followed by plating onto a highly selective medium, suggesting that this may be the most effective method for isolating *E. coli* O157:H7 from farm samples. Neither direct plating or enrichment followed by direct plating was able to detect *E. coli* O157:H7 in the bedding sample. Kudva et al. (1998) reported that wood chip bedding contributes to shorter *E. coli* O157:H7 survival times. The potential for antibiotic effects in wood chips, combined with large numbers of indigenous microflora in bedding samples, could have contributed to our inability to recover in either situation.

Recovery of low-inoculum levels of *E. coli* O157:H7 using IMS followed a similar pattern as observed for enrichment followed by direct plating, with 86% (36 of 42) of confirmed positives coming from chicken feed, TMR, and water samples. Overall, EHEC-Tek® and Reveal® for *E. coli* O157:H7 proved to be more effective than IMS for detecting low-inoculum levels of *E. coli* O157:H7. Porter at al. (1997) found that *Enterobacter agglomerans* from a variety on farm environments bound to Dynabeads® anti-*E. coli* O157 at a high rate, which could have contributed to the low isolation rates observed with the IMS method. Additionally, the presence of high levels of particulate matter likely impeded magnetic separation of beads from enrichment samples. EHEC-Tek® and Reveal® for *E. coli* O157:H7 were the most effective and reliable methods for detecting low-inoculum levels.

E. coli O157:H7 was not isolated from any of the uninoculated control samples.

This study indicates poor recovery of *E. coli* O157:H7 by direct plating using media developed to detect the organism in food from farm samples with large populations of indigenous microflora. However, it is not likely that levels of *E. coli* O157:H7 that are consistently detectable by direct plating would be present in farm samples. Rapid tests in use for detecting *E. coli* O157:H7 from food may be satisfactory in detecting low levels of the organism from farm samples.

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Part III:

Detection and Enumeration of *Yersinia enterocolitica* from Farm Animal Environments

ABSTRACT

Recovery of *Yersinia enterocolitica* from farm samples (feed, bedding, fecal samples, and water) from various animal sources (cattle, swine, and chickens) was investigated at inoculum levels of 10^4 CFU/g (high level) and 10 CFU/g (low level) of sample. High-inoculum levels of *Y. enterocolitica* were evaluated for recovery using MacConkey agar (MAC), cefsulodin-irgasan-novobiocin agar (CIN), and KV-202 agar. Low-inoculum levels of *Y. enterocolitica* were evaluated for recovery using cold enrichment in peptone sorbitol bile broth (PSBB) followed by streaking onto MAC, CIN, and KV-202. High-inoculum levels of *Y. enterocolitica* were recovered from the most sample types with CIN (7 of 8), followed by KV-202 (6 of 8), and MAC (5 of 8). CIN agar provided superior numerical recovery over KV-202 in TMR, pig fecal, chicken fecal, and pig feed sample types ($P<0.05$), but the test media did not differ significantly in their ability to recover the organism from the pig feed sample type ($P>0.05$). After enrichment, streaking to CIN provided the best recovery (19 of 46), followed by MAC (13 of 46), and KV-202 (11 of 46). These results demonstrate that direct plating onto CIN or KV-202 can be used for reasonably good detection of high levels of *Y. enterocolitica* in farm samples. However, cold enrichment in PSBB is an unreliable method for recovery of low inocula of *Y. enterocolitica* from farm animal environments.

INTRODUCTION

Yersinia enterocolitica has been shown to survive well in the diversified niches that exist in the farm animal environment. These niches must be investigated to determine the potential for contamination of animals, since the animals or their products are used as food. There is little done in the United States to monitor bacterial quality of animal feeds (Hancock et al., 1997). *Y. enterocolitica* isolates that are most often associated with human disease are most frequently isolated from swine (Kapperaud, 1991). Pathogenic isolates of *Y. enterocolitica* also have been associated with cattle (Davey et al, 1983). The organism has been found in 5.8% to 40% of pig fecal samples, with pathogenic isolates being relatively rare (Fantasia et al., 1993; Letellier et al., 1999; Sheimann, 1982; Thibodeau et al, 1999). *Y. enterocolitica* has also been shown to survive in a wide range of aquatic environments (Chao et al., 1988; Karipinar and Gonul, 1991; Terzieva and McFeters, 1991).

Media currently described by the Food and Drug Administration Bacteriological Analytical Manual (FDA, 1995) for the isolation of *Y. enterocolitica* are cefsulodin-irgasan-novobiocin agar (CIN) and MacConkey agar. Several researchers have noted that isolation on MacConkey agar is complicated because colonial morphology of *Y. enterocolitica* on MacConkey agar is similar to other bacterial species (Davey et al., 1983; Hamama et al., 1992; Bottone, 1999). CIN agar has been shown to be superior to MacConkey agar for recovery of *Y. enterocolitica* from dairy samples (Hamama et al., 1992) and cattle feces (Davey et al., 1983). A new medium, KV-202 agar, has been shown to provide recovery of *Y. enterocolitica* from enriched ground meat samples similar to that of CIN (Jiang et al., 1998). KV-202 agar employs brilliant yellow (an

alkali indicator changing from yellow to red at pH 8.0), hydrogen sulfide production, and esculin hydrolysis as a means of differentiating pathogenic *Y. enterocolitica* from non-pathogenic *Y. enterocolitica* and other species (Vichienroj, 1997). Pathogenic *Y. enterocolitica* appear as orange-red colonies surrounded by red zones with no black production from esculin hydrolysis or from hydrogen sulfide.

For the recovery of *Y. enterocolitica* from foods, the FDA BAM describes cold enrichment (10°C for 10 d) in peptone sorbitol bile broth (FDA, 1995). However, cold enrichment has been found to recover mostly non-pathogenic *Y. enterocolitica* (Van Noyen et al.,1980), but incubation temperatures above 15°C have been shown to decrease the competitive ability of *Yersinia* in relation to other Gram-negative bacteria (Shiemann and Olsen, 1984).

This investigation was undertaken to determine the efficacy of direct plating onto selective media used for foods for recovery of high levels of *Y. enterocolitica* from farm animal environments. Additionally, a food enrichment technique followed by plating on selective media was evaluated for recovery of low levels of the organism from farm animal environments.

MATERIALS AND METHODS

Samples Evaluated

Farm animal sample types evaluated were as follows: (1) Water - obtained from dairy cattle ball drinkers containing water from the Knox County municipal water system, (2) Total mixed ration dairy cattle feed (TMR) - contained corn silage, rye silage, alfalfa hay, rye hay, and protein concentrate, obtained from areas where cows had made

contact with the feed, (3) Cow fecal material - obtained from floor of dairy cattle holding pens; a mixture of feces, urine, and other matter, (4) Pig fecal material - obtained from floor of holding pens, a mixture of feces, urine, water, and other matter, (5) Chicken feed - 16% complete crumble containing 16% crude protein, 0.67% lysine, 0.33% methionine, 3% crude fat, 5% crude fiber, 4.25% calcium, 0.5% phosphorous, and 0.7% salt, obtained from chicken feeders where birds made contact with feed, (6) Chicken fecal material - contained a mixture of sawdust, feces, feathers, and other matter obtained from floor of the holding pens, (7) Pig feed - Contained corn, soybean meal, crude protein, crude fiber, crude fat, calcium, phosphorus, other vitamins and minerals, and oxytetracycline and tylosin phosphate antibiotic supplements, (8) Bedding - Sawdust material obtained from dairy cattle stalls.

All samples were obtained from University of Tennessee Agricultural Experiment Station farms. After collection, samples were immediately transported on ice to the laboratory, and held at 4°C until used (≤ 7 days). Samples were removed from the refrigerator and allowed to warm to about 20°C prior to analysis

Inoculum Preparation

Five strains of *Y. enterocolitica* used in this study were: serotype O:3, O:8, and O:20 obtained from P. Feng, Food and Drug Administration, Washington, DC, and ATCC strains 9610 and 55075 obtained from American Type Culture Collection, Rockville, MD. Strains were grown in tryptic soy broth (TSB) at 32°C and subjected to a minimum of two successive 24-hr transfers prior to inoculation into samples. Two milliters of each test strain were combined in a sterile centrifuge tube, resulting in a mixed culture containing equal proportions of the five strains. The suspension was

centrifuged at 11,068 x *g* for 10 min at 4˚C. The supernatant was decanted, cells were resuspended in 10 ml 0.5% saline, and the process was repeated. The mixed culture population (10^8 CFU/ml) was determined by surface plating onto tryptic soy agar and incubating at 37˚C for 24 hr.

Direct Plating

Media Preparation

Three media were evaluated for their ability to recover *Y. enterocoltica* from farm samples. These include Cefsulodin-Irgasan-Novobiocin (CIN), KV-202, and MacConkey (MAC) agars. Media were prepared as detailed in Appendix I.

Inoculation of Samples/Direct Plating Procedure

Twenty five grams or milliters of test samples were placed into sterile stomacher bags fitted with nylon mesh filters (Nasco). The mixed culture was serially diluted in 0.5% saline to 10^5 CFU/ml and 2.5 ml was inoculated into samples to provide an inoculum of about 10^4 CFU/g or ml of sample. For control samples, 2.5 ml of 0.5% saline was added. Inoculated samples or controls were allowed to rest for 1 hr at ambient temperature (21-24˚C). After 1 hr, 225 ml of peptone sorbitol bile broth (PSBB) was added and the suspension was pummeled in a stomacher blender for 30 sec. The resulting suspension was diluted as appropriate in 0.5% saline and surface plated (0.1 ml) onto duplicate CIN, KV-202, and MAC plates. Plates were incubated for 24 hr at 32˚C before typical *Y. enterocolitica* colonies were enumerated.

Confirmation Procedure

Typical *Y. enterocolitica* colonies from test media were streaked onto anaerobic

egg yolk agar (AEY). The isolates were incubated for 24 hr at 22-24°C before confirmation tests were performed. Isolates were tested for Gram reaction, triple sugar iron slant (TSI), urease activity, esculin hydrolysis, sucrose utilization, rhamnose utilization, Voges-Proskauer (VP; at 7 d), and citrate utilization (Simmon's formulation). Isolates that were Gram negative, produced an acid slant/butt, no acid, no gas on TSI, urease, sucrose, and VP positive, and failed to hydrolyze esculin and utilize rhamnose and citrate, were identified as *Y. enterocolitica*.

Counts obtained from direct plating studies reflect confirmed positive isolates and were transformed to log CFU/g or ml of sample. Confirmed counts were obtained using the following formula: (number of confirmed positive isolates/number of isolates tested) x number of colonies counted. For example, for 100 colonies counted with five isolates selected for confirmation and four confirmed positive, the reported count would be: (4/5) x 100 = 80 CFU/g = 1.90 log CFU/g.

Enrichment Procedure

Media Preparation

Peptone sorbitol bile broth (PSBB) was evaluated in conjunction with subsequent plating onto CIN, KV-202, and MAC. Media were prepared as detailed in Appendix I.

Inoculation of Samples/Enrichment Procedure

Test samples (25 g or ml) were added to sterile stomacher bags fitted with nylon mesh filters (Nasco). The 24-hr *Y. enterocolitica* mixed culture was diluted to 10^2 CFU/ml in 0.5% saline, and 2.5 ml of this dilution was inoculated into test samples to provide an inoculum of about 10 CFU/g or ml of sample. Control samples were inoculated with 2.5 ml of 0.5% saline. Samples were allowed to rest for 1 hr at ambient

temperature (21-24°C). After 1 hr, 225 ml of PSBB was added and the suspension was pummeled for 30 sec in a stomacher lab blender. The enrichment suspension was incubated at 10°C for 10 d. After 10 d, 0.1 ml of the enrichment was combined with 1 ml 0.5% saline in a sterile microcentrifuge tube and mixed on a vortex for 5 s. The suspension was streaked onto CIN, KV-202, and MAC, and plates were incubated for 24 hr at 32°C, before typical *Y. enterocolitica* were confirmed as described previously.

Measurement of pH

Samples were evaluated for pH using a modified AOAC method for food, placing 10 g of sample in 100 ml of distilled and reading pH after 1 hr. In addition, samples were evaluated in PSBB (225 ml in 25 g or ml). Enrichment samples were evaluated after inoculation with 0.5% saline and allowed to rest 1 hr.

Statistical Analysis

The experiments were performed in triplicate. Recovery of *Y. enterocolitica* by direct plating was statistically analyzed using the mixed procedure (PROC MIXED) of SAS (Version 7.00; SAS Institute, Cary, NC). The model was a randomized block design with replication, blocked on replication. Means were separated using LSMeans

RESULTS

The pH measurements for various farm samples are shown in Table 1. Samples ranged in pH from 5.18 (TMR) to 8.02 (cow feces) using the modified AOAC method. The pH within PSBB varied slightly, depending on sample type.

Results from recovery of high levels *Y. enterocolitica* using direct plating methods are shown in Table 2. Across all sample types, CIN recovered the organism

Table 1. The pH of various farm samples measured in diluent or enrichment broths.

Sample	Diluent/Enrichment Broth	
	Distilled Water	PSBB
Water	7.69	7.63
TMR	5.18	7.40
Cow Fecal	8.02	7.66
Pig Fecal	7.25	7.57
Chicken Feed	6.56	7.54
Chicken Fecal	7.00	7.65
Pig Feed	6.27	7.54
Bedding	7.38	7.64

Table 2. Numerical recovery of high levels (~log 4 CFU/g or ml) of *Y. enterocolitica* from farm samples[1] by direct plating onto selective media.

Sample	Population recovered (log_{10} CFU/g or ml) on[2]:		
	CIN	KV-202	MacConkey
Water	4.49 b	4.56(2) ab	4.71(2) a
TMR	4.52 a	4.23 b	4.40 ab
Cow Fecal	ND[3]	ND	ND
Pig Fecal	4.86(2) a	4.49(2) b	4.71(2) ab
Chicken Feed	4.64 a	4.47 a	4.53 a
Chicken Fecal	4.64 a	ND b	ND b
Pig Feed	4.41 a	4.10 b	4.29 a
Bedding	4.32(1) a	ND b	ND b

[1] *Y. enterocolitica* was not recovered from the controls.

[2] Data are means of 3 replications unless otherwise noted in parentheses. Means for less than 3 replications were used when the organism was not detected in one or more replications. Means in the same row followed by different letters are significantly different ($P<0.05$).

[3] Not detected; detection was not permissible due to growth of background microflora (not *Y. enterocolitica*) at levels exceeding the inoculation population of *Y. enterocolitica*.

from 18 of 24 samples, followed by KV-202 (16 of 24), and MAC (13 of 24). CIN recovered the organism from 7 of 8 sample types, followed by KV-202 (6 of 8), and MacConkey agar (5 of 8). No medium provided recovery of *Y. enterocolitica* from the cow fecal sample (P>0.05). CIN was superior to KV-202 in numerical recovery in chicken fecal, pig feed samples, water, and TMR sample types (P<0.05), but did not differ in numerical recovery from MAC agar in the pig feed, TMR, pig fecal, and chicken feed sample types (P>0.05).

Results for recovery of low inoculum levels of *Y. enterocolitica* are shown in Table 3. The combination of PSBB enrichment and streaking onto CIN provided best recovery overall (19 of 46), followed by MAC (13 of 46), and KV-202 (11 of 46). Enrichment followed by plating onto test media failed to detect the organism in pig feed, cow fecal, and bedding samples.

DISCUSSION

Recovery by direct plating was influenced by the selectivity of the medium. CIN performed favorably, recovering as well as the less selective MAC agar in samples types in which both media recovered *Y. enterocolitica* (P>0.05). MAC agar did not perform well for samples with high levels of background microflora, with the exception of the pig fecal sample. Recovery on MAC by direct plating was good overall, with the exception of chicken feed and bedding samples, where lack of selectivity of the medium prohibited recovery. MAC and KV-202 were superior to CIN in numerical recovery in the water sample type (P<0.05). KV-202 agar was difficult to read due to large black zones produced by esculin hydrolysis and hydrogen sulfide production in samples containing

Table 3. Recovery of low-inoculum levels (~10 CFU/g or ml) of *Y. enterocolitica* from various farm samples[1] after a 10 day, 10°C enrichment in peptone sorbitol bile broth followed by plating onto selective media.

Sample	Selective Medium		
	CIN	KV-202	MacConkey
Water	5/6[2]	4/6	2/6
TMR	4/6	4/6	5/6
Cow Fecal	0/6	0/6	0/6
Pig Fecal	1/6	0/6	2/6
Chicken Feed	6/6	1/6	2/6
Chicken Fecal	3/6	2/6	2/6
Pig Feed	0/4	0/4	0/4
Bedding	0/6	0/6	0/6

[1] *Y. enterocolitica* was not recovered from the controls.
[2] Values indicate number of confirmed positives/total number of observations.

large numbers of background microflora, and may have led to an underestimation of actual numbers present on the plate.

Lack of recovery by CIN in samples containing a high proportion of background microflora (cow fecal and bedding) can be attributed to microorganisms that produce colonies similar to *Y. enterocolitica* on CIN. Davey et al. (1983) reported that strains of *Citrobacter freundii*, *Serratia marcescens*, other *Serratia* spp., and a strain of *Proteus vulgaris* fell under this category. Indeed, biochemical testing revealed that *C. freundii*, and *Serratia* spp. were among those picked as presumptive *Y. enterocolitica* on this media.

Enrichment proved to be effective for the majority of the samples with comparatively low amounts of indigenous microflora. The disparity in detection of the organism in pig feed and chicken feed, both dry feeds, may lie in the fact that the chicken feed is ground. The ground chicken feed may have allowed for interaction with moisture in the air, facilitating survival.

This study indicates poor recovery of *Y. enterocolitica* by direct plating using media developed to detect the organism in food from farm samples with large populations of indigenous microflora. However, it is not likely that levels of pathogenic *Y. enterocolitica* consistently detectable by direct plating would be present in farm samples. Cold enrichment in PSBB followed by plating onto selective media is an unreliable method for detecting low levels *Y. enterocolitica* from farm samples with large populations of indigenous microflora.

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Part IV:

Conclusion

The results from this research indicate that current techniques for the detection of *E. coli* O157:H7 and *Y. enterocolitica*, which are primarily intended for use in foods, may be adapted for use in the farm environment. However this research illustrated the need for more effective methodology in samples with large numbers of background microflora.

Results from the recovery of high- and low-inoculum levels of *E. coli* O157:H7 from farm animal environments can be summarized as follows:

1. Media incorporating additional selective agents were more effective at recovery of *E. coli* O157:H7 from farm samples, although numerical recovery was often reduced.
2. Rainbow® agar O157 was able to recover *E. coli* O157:H7 by direct plating from the most sample types, but CT-SMAC provided better numerical recovery in 5 of 6 samples in which both media recovered the organism.
3. In general, enrichment in a less selective medium followed by plating onto a highly selective medium was more effective at recovering low levels of *E. coli* O157:H7 from farm samples.
4. EHEC-Tek® was the most effective and reliable method for detecting low levels of *E. coli* O157:H7 in farm samples.

Results from the recovery of high- and low-inoculum levels of *Y. enterocolitica* from farm animal environments can be summarized as follows:

1. CIN performed favorably in recovering high levels of *Y. enterocolitica*, recovering as well as less selective MAC in samples where both recovered

the organism.

2. Difficulty in reading KV-202 may have lead to an underestimation of numerical recovery on the medium and contributed to the inabilty to recover *Y. enterocolitica* from enrichment samples.

3. Enrichment in PSBB followed by plating onto CIN provided the best recovery of low levels of *Y. enterocolitica* in samples with the least amount of background microflora, but all methods were ineffective in recovering the organism from highly contaminated samples.

Appendix

MEDIA PREPARATION

Cefsulodin-Irgasan-Novobiocin Agar (CIN)

Prepare using Difco CIN agar base and Difco CIN selective supplement and follow manufacturers' instructions.

Cefixime-Tellurite Sorbitol MacConkey Agar (CT-SMAC)

Prepare using Oxoid sorbitol MacConkey agar supplemented with Cefixime-Tellurite supplement (Dynal, Lake Success, NY) and follow manufacturers' instructions.

***Escherichia coli* (EC) Broth, modified, with novobiocin (mEC+N)**

Per liter of medium:

Tryptone, 20 g

Bile salts No. 3, 1.12 g

Lactose, 5.0 g

Potassium phosphate (dibasic), 4.0 g

Potassium phosphate (monobasic), 1.5 g

Sodium chloride, 5.0 g

Water, 1.0 L

Sterilize in autoclave at 121˚C for 15 min, cool (~50˚C), and add filter sterilized novobiocin to result in a concentration of 0.02 g/l in the final medium.

EHEC Enrichment Broth (EEB)

Per liter of medium:

Trypticase soy broth, 30 g

Bile salts No. 3, 1.12 g

Potassium phosphate (dibasic), 1.5 g

Deionized water, 1 L

Sterilize in autoclave at 121˚C for 15 min, cool (~50˚C), and add the following filter sterilized antibiotic solutions immediately prior to addition to sample, to give the final concentration indicated per liter of medium.

Novobiocin, 20 mg

Cefixime, 0.05 mg

Cefsulodin, 10 mg

Vancomycin, 8 mg

Hemorrhagic Colitis Agar (HC)

Per liter of medium:

Tryptone, 20 g

Bile salts No. 3, 1.13 g

Sodium chloride, 5 g

Sorbitol, 20 g

MUG reagent (Oxoid), 0.1 g

Bromcreosol purple, 0.015 g

Agar, 15 g

Deionized water, 1 L

Sterilize in autoclave at 121˚C for 15 min.

KV-202 Medium

Per liter of medium:

Peptone, 20 g

Yeast extract, 2.0 g

Sodium desoxycholate, 0.7 g

Sodium chloride, 1.0 g

Magnesium sulfate, 0.01 g

Ferrous sulfate, 0.1 g

Dulcitol, 20.0 g

Pyruvic acid, 2.0 g

Esculin, 1.0 g

Brilliant yellow, 0.5 g

Agar, 15.0 g

Deionized water, 1 L

Boil to dissolve completely, adjust pH of medium to 7.4. Sterilize in autoclave at 121˚C, 15 psi, 15 min. Allow medium to cool to 50˚C. Add the following filter sterilized antibiotic solutions immediately prior to addition to sample, to give the final concentration indicated per liter of media:

Cefsulodin, 15 mg

Novobiocin, 2.5 mg

Irgasan, 4 mg

The antibiotics can be obtained together from Oxoid (*Yersinia* selective supplement; Cat. No. SR109E).

MacConkey Agar

Prepare using Difco MacConkey agar CS (Cat. No. 1818-17-1) and follow manufacturers' instructions.

Modified eosin methylene blue agar (MEMB)

Per liter of medium:

Peptone,10 g

Potassium phosphate dibasic, 2.0 g

Eosin Y, 0.4 g

Methylene blue, 0.065 g

Sorbitol, 10 g

Sodium chloride, 10 g

Agar, 15 g

Sterilize in autoclave at 121˚C for 15 min, cool to 50˚C. Add the following filter sterilized antibiotic solution to give the final concentration indicated per liter of media:

Novobiocin, 0.02 g

Peptone sorbitol bile broth (PSBB)

Per liter of medium:

Na_2HPO_4, 8.23 g

$NaH_2PO_4 \cdot H_2O$, 1.2 g

Bile salts No. 3, 1.5 g

Sodium chloride, 5 g

Sorbitol, 10 g

Peptone, 5 g

Deionized water, 1 L

Dispense into portions, sterilize in autoclave at 121˚C for 15 min.

Rainbow® agar O157

Prepare using Rainbow® agar O157 (Biolog, Hayward, CA). Sterilize in autoclave at 121˚C at 15 psi for 10 min. Add the following filter sterilized antibiotic solutions after cooling media to 50˚C, to obtain the concentration indicated per liter of media:

Tellurite, 0.0008 g

Novobiocin, 0.01 g

Sorbitol MacConkey agar (SMAC)

Prepare using sorbitol MacConkey agar (Oxoid) and follow manufacturers' instructions.

Tryptic soy broth, modified, with novobiocin (mTSB+N)

Per liter of medium:

Tryptic soy broth, 30 g

Bile salts No. 3, 1.12 g

Dipotassium phosphate, 1.5 g

Deionized water, 1 L

Dispense into portions, sterilize in autoclave at 121°C for 15 min, and cool to room temperature. Add the following filter sterilized antibiotic solution immediately prior to addition to sample, to obtain the concentration indicated per liter of medium:

Novobiocin, 0.02 g

VITA

Christopher Aaron Kiefer was born on January 11, 1975 in Toledo, Ohio. He graduated from Farragut High School in Knoxville, Tennessee in May 1993. He was awarded a B.S. in Nutrition and Food Science from Auburn University in June 1997. He was awarded a M.S. in Food Science and Technology from the University of Tennessee, Knoxville in May 2000.

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